獸醫學碩士 學位論文

Establishment of a new molecular method for influenza A virus detection using improved generic primers and concentration of viral RNP

개선된 제네릭 프라이머와 새로운 바이러스 RNP 복합체 농축을 통한 A형 인플루엔자 바이러스 분자적 진단 기법 확립

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Establishment of a new molecular method for influenza A virus detection using improved generic primers and concentration of viral RNP

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Abstract

Establishment of a new molecular method for influenza A virus detection using improved generic primers and concentration of viral RNP

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The mismatch rate and positions of primers and templates as well as the copy numbers of target genes in specimens may affect the sensitivity of polymerase chain reaction (PCR)-based diagnostics. To date, various

generic primer sets to detect influenza A viruses (IAVs) have been reported; however, their mismatch rates have not been extensively evaluated. In this study, we verified the nucleotide identities of reported primer sets with 3,441 complete coding regions of matrix genes. According to the results, none of compared primers showed 100% identity to more than 86% of the compared genes.

Therefore, we designed a new degenerate primer set with 100% identity to more than 98% of compared genes and compared the amplification efficiency of the primer set using SYBR-based real-time reverse transcription PCR (SYBR-RT-qPCR) with TaqMan probe-based RT-qPCR. The analytical sensitivity of each RT-qPCR method was assessed by determining the smallest amount that can be detected from the serial dilutions of the IAV's viral RNAs. According to our results, both RT-qPCR methods showed similar analytical sensitivity (1.37 EID_{50}) when performed with viral RNAs of wild-type PR8 virus, whereas our new SYBR-RT-qPCR method showed about ten-fold higher analytical sensitivity (7.89 EID_{50}) than that of Taqman probe-based RT-qPCR method (78.9 EID_{50}) when performed with viral RNAs of recombinant PR8 virus which has a single nucleotide mismatch in the 5^{th} position from the 3' end of Spackman's reverse primer. In addition, we compared the clinical sensitivity, specificity, and positive predictive value between our new SYBR-RRT PCR method and Taqman probe-based RT-qPCR in 293 fecal samples of migratory birds

collected from the drainage basin of the the Geum-gang river. The clinical sensitivities of the two RT-qPCR methods were similar as 100%, while the clinical specificity and positive predictive value of SYBR RT-qPCR were 95.41%, and 43.48%, respectively, both of which are higher than 85.16%, and 19.23%, of Taqman probe-based RT-qPCR.

Furthermore, we successfully increased the sensitivity of SYBR-RT-qPCR by concentrating the complex of viral RNA and nucleoprotein (RNP) in allantoic fluid and feces reacting with Triton X-100, anti-nucleoprotein mouse monoclonal antibodies, and anti-mouse immunoglobulin antibody-conjugated magnetic beads. The concentration of viral RNA in the eluate was significantly increased by 64-fold compared to the conventional RNA extraction method by using our RNP-concentrating method. In addition, we verified that our RNP concentration method could detect IAVs below the detection limit of the conventional RNA extraction method. Thus, the improved generic primer set and RNP concentration method together can be applied as useful tool for sensitive detection of IAVs.

Keyword: influenza A virus, matrix gene, generic primer, real-time reverse transcription polymerase chain reaction, concentration of the complex of viral RNA and nucleoprotein

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Abbreviations

HA	Hemagglutinin
NP	Nucleoprotein
NA	Neuraminidase
M	Matrix
IAV	Influenza A virus
vRNP	Viral ribonucleoprotein
AIV	Avian influenza virus
LPAIV	Low-pathogenic avian influenza virus
ECE	Embryonated chicken egg
MDCK cells	Madin-Darby canine kidney cells
293T	Human embryonic kidney cells
EID_{50}	50% chicken embryo infectious dose
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RT-qPCR	Reverse transcription-qPCR
IRD	Influenza research database
PR8	A/Puerto Rico/8/34
vRNA	Viral genomic RNA
PBS	Phosphate buffered saline

1. Introduction

Influenza A viruses (IAVs) are enveloped, segmented, single-stranded, negative-sense RNA viruses of the family *Orthomyxoviridae* and can be divided into types A, B, and C. Avian IAVs (AIVs) are further divided into 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes. Wild aquatic birds serve as reservoirs of all known subtypes, and fatal direct transmission from birds to humans has necessitated monitoring of AIVs among wild and domestic birds and environmental samples (Claas et al., 1998; Fouchier et al., 2005; Webster et al., 1992). Virus isolation using embryonated chicken eggs (ECEs) and MDCK cells has been the golden standard for monitoring of avian and mammalian IAVs; however, these methods are time consuming and require special facilities to control biohazards. Reverse transcription polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification, and real-time RT-PCR (RT-qPCR) have been developed as alternative methods for the detection of IAVs (Ellis and Zambon, 2001; Fouchier et al., 2000; Lau et al., 2004; Spackman et al., 2002; Starick et al., 2000; Trani, 2006; Van Borm et al., 2007). Among those molecular diagnostic techniques, RT-qPCR, nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) are most commonly used.

Nucleic aicd sequence-based amplification (NASBA) is an isothermal nucleic acid amplification method that uses a mixture of avian myeloblastosis virus reverse transcriptase, RNAse H, T7 DNA dependent RNA polymerase, and two specially designed oligo-nucleotides (Compton, 1991). NASBA is highly sensitive and specific diagnostic method that can amplify target RNA sequence to more than 10^9 copies in 90-120 minutes. Moore et al. evaluated NASBA for rapid detection of IAVs in 389 clinical samples obtained across Wales during the 2003/2004 season. The assay demonstrated the detection limit of 0.01 TCID50 or 10-100 copies of vRNA and high assay sensitivity of 95% compared to direct immunofluorescence (IF) (Moore et al., 2004). Although the NASBA assay is highly sensitive, it has not been widely used because of difficulties in the preparation of the master mix and the high cost of commercial kits. Loop-mediated isothermal amplification-based assay (LAMP) is an isothermal nucleic acid amplification approach that has been evaluated for detection of several viruses including influenza virus. LAMP uses a novel DNA polymerase with high strand displacement activity and four primers including two looping primers and two stripping primers that recognize six distinct regions on the viral cDNA. Poon et al. detected influenza A/WSN/33 virus with analytic sensitivity of 10^{-3} PFU per reaction by amplification for 2 hours (Poon et al., 2005). Reverse transcriptase is added to the reaction for reverse transcription performed with primers. The RT-LAMP assay performed

better than a WHO-approved RT-PCR assay while testing 239 clinical samples. It demonstrated up to 10-fold higher sensitivity compared to RT-PCR method with an analytical sensitivity of 0.1 $TCID_{50}$/mL (Parida et al., 2011). Although the simplicity as well as high sensitivity and specificity makes it suitable for field surveillance and diagnosis in developing countries, the difficulties in optimization of the primer design to target six distinct regions still exist. Real-time RT-PCR has been traditionally and most frequently used for detecting influenza A viral genomes and for virus subtyping. The method employs oligonucleotide probes conjugated with a fluorophore or intercalator for staining PCR products. One example is the TaqMan probe method, in which the oligonucleotide probe is conjugated with a fluorophore and quencher at the 5′ and 3′ terminal region, respectively. The degradation of the probe by the exonuclease activity of Taq polymerase causes the separation of the fluorophore from the quencher and makes fluorescence detectable. SYBR green is also used as an intercalating dye in RT-qPCR. It strongly interacts with double-stranded DNA. Modifications to real-time RT-PCR methods have been applied to decrease the time required for both the identification of the virus subtype and its sequencing. For example, Spackman et al. used an one-step RT-qPCR system for the detection of AIVs and determination of the H5 or H7 subtype (Spackman et al., 2002). For rapid diagnosis, disease confirmation and large-scale surveillance, real-time RT-PCR has worked well. Therefore,

RT-qPCR has been considered a golden standard assay for influenza diagnosis.

Although matrix genes are often targets in molecular diagnosis because of their conservation among IAVs, reported primer sets have not been evaluated extensively by comparing nucleotide sequences of primers with sufficient numbers of matrix genes from influenza viruses.

For molecular diagnosis, small amounts of specimens are used; however, the use of concentrated samples may increase sensitivity. Although various concentration methods have been reported, these previous methods cannot be applied for rapid molecular diagnosis (Arora et al., 1985; Coloma et al., 2009; Heyward et al., 1977; Hirst, 1941). Recently a simple method to purify virions using anti-nucleoprotein (NP) antibody-bound magnetic beads was developed, and purified virions were directly used for RT-qPCR without RNA extraction (Dhumpa et al., 2011).

Therefore, in this study we evaluated several reported primer sets by comparing corresponding nucleotide sequences in the matrix database (www.flu.lanl.gov/search/; July 1, 2007). We then developed a degenerate primer set that showed high nucleotide matching rates ([number of 100% identical matrix genes / total number of matrix genes containing a corresponding primer sequence] × 100) with diverse matrix genes in the database. We applied the improved primer set to SYBR Green (SYBR)-based RT-qPCR for detection of IAVs and compared the results with

TaqMan probe-based RT-qPCR using various IAVs and recombinant PR8 viruses with nucleotide mismatches in Spackman's forward or reverse primers, which had been used for diagnosis of AIVs in Korea (Spackman et al., 2002). In addition, we targeted the relatively conserved NP protein for purification and concentration of the complex of viral genomic RNA and NP (RNP). After treatment with the optimal concentration of Triton X-100, we treated samples with anti-NP mouse monoclonal antibodies (mAbs) to bind to exposed RNP and purified the RNP with anti-mouse immunoglobulin goat antibody-conjugated magnetic beads.

2. Materials and Methods

Virus, eggs, and cells

The influenza viruses A/chicken/Korea/KBNP-0028/2000(H9N2) (0028), A/Purerto Rico/8/1934(H1N1) (PR8), A/Singapore/1/57 (H2N2), A/duck/Ukraine/1/63 (H3N8), A/duck/Czechoslovakia/56 (H4N6), A/duck/Hong Kong/820/80 (H5N3), A/shearwater/Australia/1/72 (H6N5), A/duck/Hong Kong/301/78 (H7N1), A/turkey/Ontario/6118/68 (H8N4), A/turkey/Wisconsin/1/66 (H9N2), A/Chicken/Germany/N49 (H10N7), A/duck/England/56 (H11N6), A/duck/Memphis/546/74 (H11N9), A/duck/Alberta/60/76 (H12N5), and A/gull/Maryland/704/77 (H13N6); a Newcastle disease vaccine strain (La Sota); and an infectious bronchitis virus (SNU-11045) were propagated in 10-day-old specific pathogen-free (SPF) ECEs (Charles River Laboratories, USA) by incubation for 3 days at 37°C after inoculation with the virus via the allantoic cavity route. Field isolates of infectious bursal disease virus (SNU16001 in cecal tonsils) and reticuloendotheliosis virus (REV, SNU16008 in thymus) were diagnosed by our laboratory, and homogenized infected tissues were kept at -70°C until use.

Chicken embryo kidney (CEK) cells were cultured using Eagle's minimum essential medium (MEM; Life Technologies Co., NY, USA) supplemented with 5% fetal bovine serum (FBS; Life Technologies Co.).

293T cells were purchased from the American Type Culture Collection (ATCC, VA, USA) and maintained in Dulbecco's modified Eagle's medium (Life Technologies Co.) supplemented with 5% FBS. 293T cells were used for the generation of recombinant viruses by reverse genetics.

Titration of virus

Each virus was diluted in sterile phosphate-buffered saline (PBS) using 10-fold serial dilutions, and diluted viruses were inoculated into four 10-day-old SPF ECEs. At 3 days postinoculation, allantoic fluid was harvested and tested for hemagglutination with 1% chicken red blood cells. The 50% embryo infectious dose (EID_{50}) was calculated by the Spearman-Karber method (Hamilton et al., 1977).

Collection of matrix genes and comparison of nucleotide sequences

We collected 3,441 complete matrix (M) genes from IAVs using the IVDB database (http://influenza.genomics.org.cn). The nucleotide sequences of reported primers were queried to find and count 100% matched identical sequences using the "Filter" option in Excel 2014 (Microsoft Co., Redmond, WA, USA). Mismatched sequences were counted manually, and the identity rate of a given primer was represented as a percentage by calculation of the number of 100% identical sequences of the

total number of compared sequences (3,441).

Design of a new primer set for SYBR-based RT-qPCR (SYBR-RT-qPCR)

We selected 100 complete matrix genes from unrelated influenza viruses and compared the nucleotide sequences. We selected the most conserved two regions, 7–27 ('A' of the start codon, ATG, was numbered 1, and upstream nucleotides were numbered as negatives; the same region as Fouchier's forward primer) and 207–225. The nucleotide sequences of the two regions were compared with matrix genes of the database as described above, and mismatched sequences were classified into groups. Degenerate primers reflecting sequence variations are listed in Table 1.

RNA extraction and RT-qPCR

Viral RNA was extracted with a Viral gene-spin kit (iNtRON Biotechnology, Seongnamsi, Korea) or Qiagen RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA was eluted from the QIA spin column in a final volume of 50 μL sterilized DEPC-treated distilled deionized water (DEPC-DDW). RT-qPCR was performed on an ABI StepOne instrument (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBRGreen RT-PCR kit and QuantiTect Taqman probe RT-PCR kit (Qiagen Co.) according to the manufacturers' protocols. For SYBR-RT-qPCR, the 10-μL reaction mixture consisted of 5

μL SYBR reaction mix, 0.2 μL RT mix, 0.2 μL (10 pmol) each primer, 3.4 μL DEPC-DDW, and 1 μL diluted RNA template. For TaqMan probe-based RT-qPCR (TaqMan-RT-qPCR), the 10-μL reaction mixture consisted of 5 μL reaction mix, 0.2 μL RT mix, 0.2 μL (10 pmol) each primer, 0.2 μL specific probe (10 pmol), 3.4 μL DEPC-DDW, and 1 μL diluted RNA template. The reaction conditions were as follows: for SYBR-RT-qPCR, 46°C for 30 min and 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 46°C for 20 s, and 68°C for 25 s, and a melt curve was generated from 60°C to 95°C; for TaqMan-RT-qPCR, 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 10 s, 55°C for 30 s, and 72°C for 10 s. The threshold was adjusted using the default settings for the StepOne software v2.3.

Site-directed mutagenesis of PR8 virus M gene and recombinant virus generation

In order to generate two recombinant viruses with mismatches at the eighth nucleotide (from the 3′ end) of the forward primer and the fifth (from the 3′ end) and seventh (from the 5′ end) nucleotides of the reverse primer (Fig. 1.), site-directed mutagenesis was implemented by using a Muta-direct Site Directed Mutagenesis Kit (iNtRON) according to the manufacturer's protocol (5′-AAAGATGAGTCTTCTAACAGAGGTCGAAACGTACGTA-3′ / 5′-

TACGTACGTTTCGACCTCTGTTAGAAGACTCATCTTT-3′; F 5′-AAGCCGAGATCGCACAGAAACTTGAAGATGTCTTTGC-3′ / R 5′-GCAAAGACATCTTCAAGTTTCTGTGCGATCTCGGCTT-3′). The nucleotide sequence of the insert was confirmed by sequencing with cmv-SF (5′-TAAGCAGAGCTCTCTGGCTA-3′) and bGH-SR (5′-TGGTGGCGTTTTTGGGGACA-3′) primers.

Two mutant PR8 viruses (MF1;mPR8-F-$C8_3A$ and G80A;mPR8-R-$C7_5T$-$A5_3G$) were generated by Hoffmann's reverse genetics system (Fig. 2.), as described previously (Hoffmann et al., 2000). Each mutated M gene plasmid was mixed with seven internal gene plasmids for PR8 (300 ng of each plasmid plus Lipofectamine2000 Plus reagents [Life Technologies Co.]) and transfected into 293T cells as described previously, with some modifications (Kim et al., 2014). After overnight incubation, 1 mL Opti-MEM (Life Technologies Co.) and 0.5 mg/mL L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) were added to transfected 293T cells. After 24 h, the culture medium was harvested, and 200 μL medium was injected into 10-day-old SPF ECEs via the allantoic cavity route. Three days after inoculation, the allantoic fluid was harvested and checked for viral growth using HA assays, as recommended by the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance. All mutant viruses were confirmed by RT-PCR and sequencing (Macrogen Inc., Seoul, Korea).

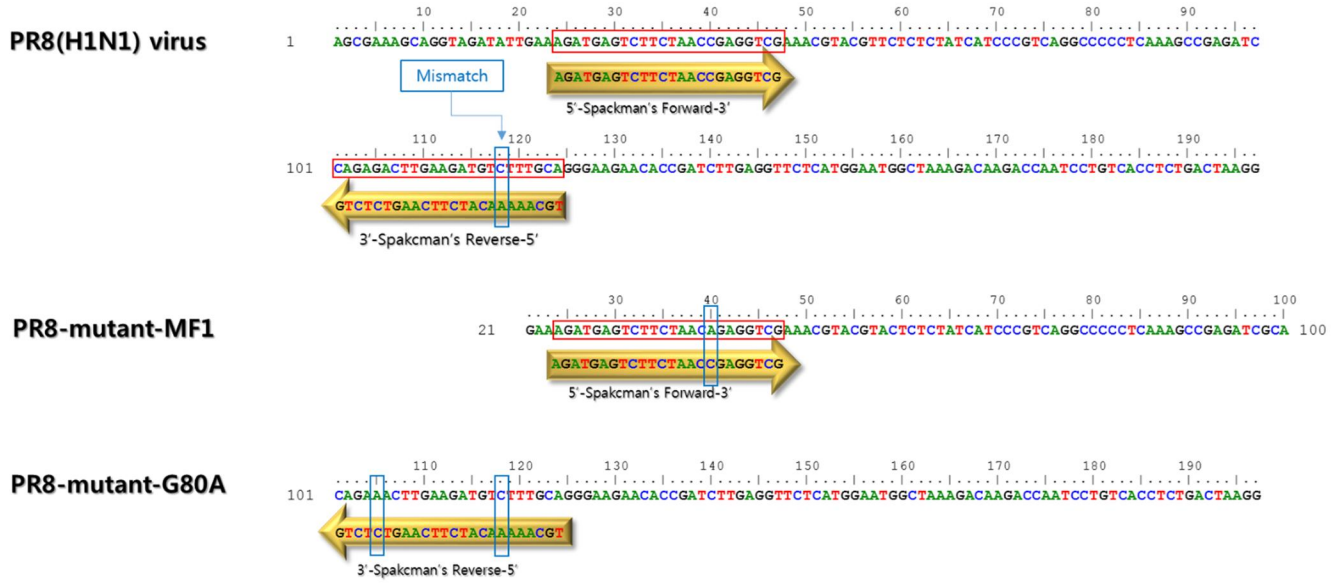

Fig. 1. Locations of nucleotide mismatches between Spackman's primers and M genes of wild-type or recombinant PR8 viruses.

The locations of the nucleotide mismatches between the primer set and M gene are presented. The two recombinant PR8 (H1N1) viruses were generated to possess single-nucleotide mismatches near the 3' end of the primer-binding region of each virus. The recombinant virus, MF1; mPR8-F-$C8_3A$ has a mismatch in the 8^{th} nucleotide from 3' end and G80A; mPR8-R-$C7_5T$-$A5_3G$ has an intended mismatch in the 5^{th} nucleotide from 3' end as well as a pre-existing mismatch in the 7^{th} nucleotide from 5' end of the

primer-binding region..

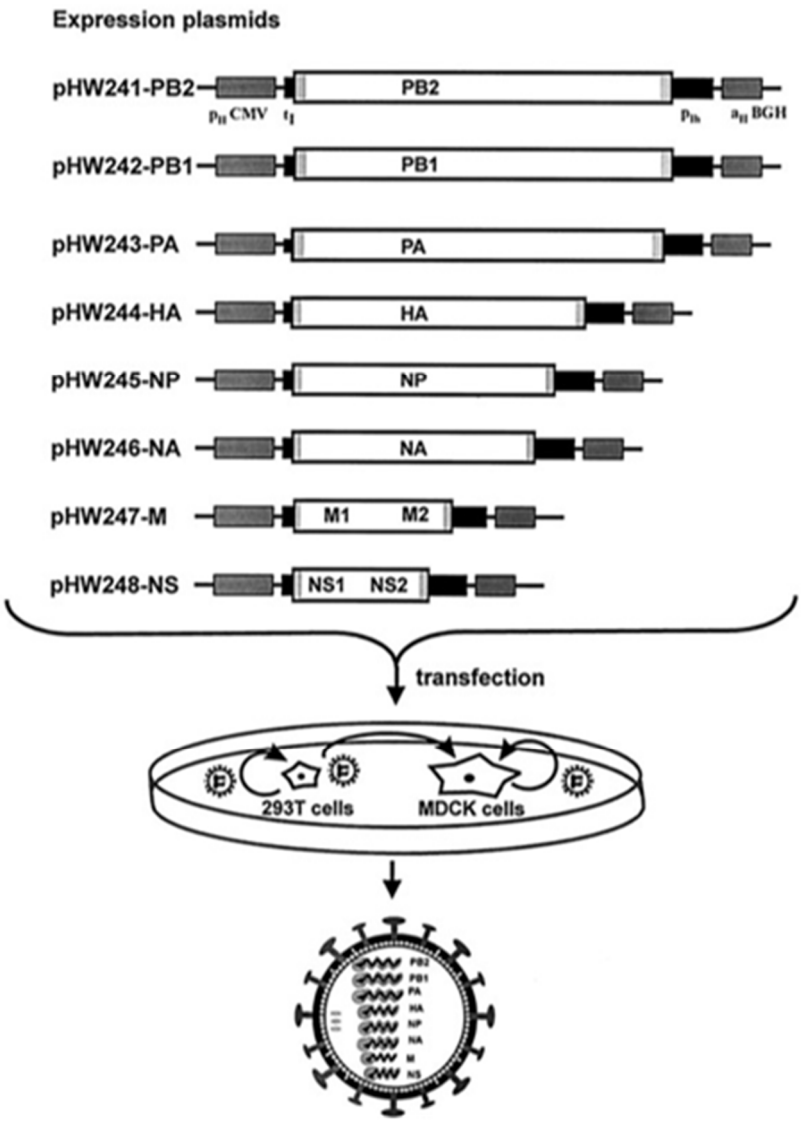

Fig. 2. Generation of recombinant influenza virus using eight-plasmid reverse genetics system

Hoffman's eight-plasmid reverse genetics system was used to generate recombinant PR8 viruses. Viral cDNAs of eight genomic segments were inserted into pHW2000 vectors and the vectors containing viral cDNAs were introduced into 293T cells by transfection. After 72 hours of incubation, the supernatant which is abundant with virions are collected.

(Adapted from E. Hoffman et al., 2000, *PNAS*, vol. 97, 6108-6113)

Sensitivity and specificity of SYBR- and TaqMan-RT-qPCR

To compare the sensitivity of SYBR- and TaqMan-RT-qPCR, extracted viral RNA was diluted 10-fold (from 10^{-1} to 10^{-10}), and the detection limit was calculated by dividing the titer (EID_{50}) of each virus by the dilution factor. To verify the specificity of the SYBR-RT-qPCR method in this study, we performed SYBR-RT-qPCR with RNA samples prepared from a panel of IAVs and avian RNA viruses. If necessary, the presence and absence of the correctly sized amplicon at the end point of positivity and negative control were confirmed by gel electrophoresis on 2% agarose gels and visualization using a UV-transilluminator.

Optimization of the concentration of Triton X-100

To optimize the concentration of Triton X-100 that exerts inactivation activity, 0.3% and 0.5% Triton X-100 (Sigma-Aldrich) allantoic solutions were prepared by mixing 30 or 50 μL of 10% Triton X-100 in PBS with 970 μL KBNP-0028-infected allantoic fluid. After incubation at room temperature for 30 min, samples were diluted by 10-fold to 10^{-7}, and 10 μL of each diluted solution was added to CEK cells cultured in 96-well plates. Four wells for each diluted solution were tested for virus replication using plate agglutination tests with 0.5% chicken red blood cells (RBCs) and culture medium. The same volumes of RBCs and culture medium were mixed for hemagglutination.

Concentration of RNP in allantoic fluid and feces

We developed a novel method for concentration of viral RNPs using anti-NP mAb and anti-IgG antibody-conjugated magnetic bead (Fig. 3.). We added 0.3, 0.5, 0.7, 1, or 1.5 mL of 10% Triton X-100 to 5 mL allantoic fluid, or to 5 mL of the 10% PBS suspension of chicken feces which contains PR8 influenza A virus particles. Then, 200 unit of RNase inhibitor was added followed by treatment of anti-NP mouse monoclonal antibodies (anti-NP-mAbs; Santa Cruz Biotechnology Inc., Texas, U.S.A.) and anti-mouse immunoglobulin G goat antibody-conjugated magnetic beads (aMBs; Dynabeads, Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) were added and incubated for 30 min at room temperature with mild mixing. The RNP-anti-NP-mAb-aMB complex was separated with a magnet, and the solution was removed. One or two times of washing with PBS was conducted to wash away other contaminants from the suspension. Next, 250 μL of RNA lysis buffer from the iNtRON Viral gene spin kit was added to the separated complex, and RNA was purified according to the manufacturer's protocol. Then, the purified RNA was subjected to SYBR-RRT PCR.

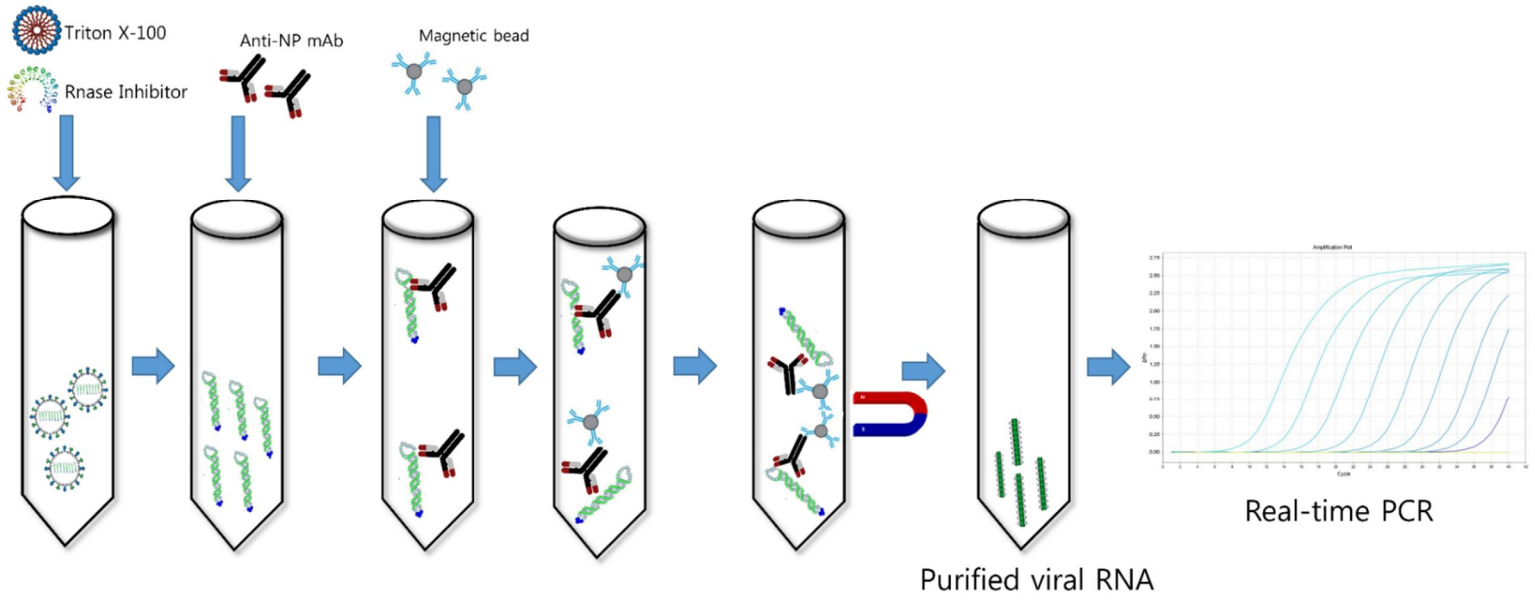

Fig. 3. Schematic diagram of the flow of RNP concentration method

Triton X-100, a non-ionic detergent, is added to the fecal suspension containing influenza A virus-like particles to disrupt the integrity of the envelope and expose viral RNPs to the exterior. RNase inhibitor is used to inhibit the decomposition of viral RNAs by RNases. Then, anti-NP monoclonal antibody and secondary antibody-conjugated magnetic beads are treated to the suspension. While a strong magnetic bar is attracting the RNP-antibody-magnetic bead complex, remove the supernatant and wash the tube with PBS. Finally, viral RNAs are extracted from the RNP-antibody-magnetic bead complexes and are used in SYBR-RRT PCR.

Enhanced detection sensitivity by RNP concentration method

To verify the practicality of RNP concentration method for the enhancement of detection sensitivity of SYBR-RT-qPCR in the field samples, we compared the detection ability of the examination between conventional RNA extraction method and our RNP concentration method. The allantoic fluid containing PR8 virus was diluted in 5 mL of 10% fecal suspension to final dilutions of 10^{-6} and 10^{-7}. In each dilution, conventional methods for RNA extraction and RNP concentration were performed as above for the extraction of viral RNA. The extracted RNAs were examined by SYBR-RT-qPCR. Whether the influenza virus was detected by the examination was decided by the melt curve analysis.

Comparison of M gene-positive rate and diagnostic accuracy between SYBR-RT-qPCR and Taqman-RT-qPCR for detection of AIVs in fecal samples

To compare the sensitivity and specificity of SYBR-RT-qPCR and Taqman-RT-qPCR methods, two RT-qPCR methods were applied simultaneously to the 600 fecal samples of migratory birds collected in the drainage basin of the the Geum-gang river, Chungnam, Korea. The specimens were pooled by 5 samples in one tube. 120 pooled fecal samples were suspended in PBS in a volume ratio of 1:5. Fecal suspensions were

strongly mixed by vortexer and centrifuged at 3, 000 rpm for 10 minutes. Following centrifugation, the supernatant of each sample was collected and viral RNA was extracted from the supernatant using iNtRONTM Viral gene spin kit. RNAs were subjected to both of the RT-qPCR methods using QIAGEN Quantitect RT-PCR kit according to the manufacturer's protocol. For virus isolation, 1ml of the supernatant from each pooled fecal sample was treated with antibiotics and inoculated to three SPF embryonic chicken eggs by 0.2ml/egg. After 72 hours of incubation, allantoic fluid was collected and subjected to HA test. Additionally, RT-PCR was performed on the HA-positive samples for the confirmation of the presence of IAV.

The measures of diagnostic accuracy were evaluated using virus isolation method as a "gold standard" and were calculated as follows:

$$\text{sensitivity} = \frac{\text{number of samples positive in RT-qPCR and virus isolation}}{\text{number of samples positive in virus isolation}} \times 100\ (\%)$$

$$\text{specificity} = \frac{\text{number of samples negative in RT-qPCR and virus isolation}}{\text{number of samples negative in virus isolation}} \times 100\ (\%)$$

$$\text{positive predictive value} = \frac{\text{number of samples positive in RT-qPCR and virus isolation}}{\text{number of samples positive in RT-qPCR}} \times 100\ (\%)$$

$$\text{negative predictive value} = \frac{\text{number of samples negative in RT-qPCR and virus isolation}}{\text{number of samples negative in RT-qPCR}} \times 100\ (\%)$$

3. Results

Comparison of nucleotide sequence identities of reported and new generic primer sets to target viral genes in the database

We collected 3,441 matrix genes from the database and compared the nucleotide sequences of reported primer sets. Of the compared primer sets, Starick's and Lau's primer sets showed relatively high 100% identity rates for both primers, i.e., 85.4%/85.1% and 82.3%/84.0%, respectively (Table 1). The other primer sets showed relatively low identity rates, which would be expected to reduce the efficiency of PCR-based diagnosis. Therefore, we designed improved forward and reverse degenerate primers for SYBR-RT-qPCR (Table 1). The forward primer (MF1) was located in the same position as Fouchier's forward primer (7–29) but contained two degenerate nucleotides at positions 6 and 9 from the 5′ end. The reverse primer (MR1) was located at 225–207 and contained two degenerate nucleotides at positions 10 and 13 from the 5′ end. Therefore, the 100% identity rates of MF1 and MR1 were expected to be 98.2% and 98.6%, respectively (Table 1). The high identity rate of the new generic primer set was expected to increase the sensitivity of the RT-qPCR assay.

Table 1. Evaluation of generic primer quality based on nucleotide sequence identity with matrix genes in the database [a].

Reference	Primer			100% identity rate (%)
	Direction	Location[a]	Sequence	
Ellis *et al.*	Forward	202–220	GTGCCCAGTGAGCGAGGAC	84.8 (2917/3441)
	Reverse	614–597	ATCTCCATGGCCTCTGCT	0.3 (9/3441)
Fouchier *et al.*	Forward	7–29	CTTCTAACCGAGGTCGAAACGTA	84.0 (2,890/3,441)
	Reverse	251–228	AGGGCATTTTGGACAAAKCGTCTA	69.3 (2,383/3,441)
Lau *et al.*	Forward	7–29	Fouchier′s forward	84.0 (2,890/3,441)
	Reverse	251–228	ARGGCATTYTGGACAAAKCGTCTA	83.0 (2,855/3,441)
Spackman *et al.*	Forward	-2–22	AGATGAGTCTTCTAACCGAGGTCG	52.4 (1,803/3441)
	Reverse	99–76	TGCAAAAACATCTTCAAGTCTCTG	4.0 (137/3441)
Starick *et al.*	Forward	224–240	AGCGTAGACGCTTTGTC	85.4 (2,937/3,441)
	Reverse	824–808	GACGATCAAGAATCCAC	84.5 (2,908/3,441)
Trani *et al.*	Forward	7–29	Fouchier′s forward	84.0 (2,890/3,441)
	Reverse	154–133	GGATTGGTCTTGTCTTTAGCCA	77.9 (2,680/3,441)
Van Borm *et al.*	Forward	3–20	GAGTCTTCTAACCGAGGT	52.9 (1,820/3,441)
	Reverse	761–743	GATCACTTGAATCGCTGCA	38.5 (1,329/3,441)
This study	Forward (MFN1)	7–27	CTTCTRACMGAGGTCGAAACG	98.2 (3,374/3,441)
	Reverse (MRON1)	225–207	CTGCAGTCCYCGYTCACTG	98.6 (3,394/3,441)

[a] IVDB: http://influenza.genomics.org.cn/search/complexQuery.jsp

[b] The primer locations are numbered from the start codon of the matrix gen

Comparison of the sensitivity, specificity and detection limit of SYBR-RT-qPCR with those of TaqMan-RT-qPCR

The specificity of SYBR-RT-qPCR was assessed using a panel of IAVs composed of various HA and NA subtypes and avian RNA viruses, such as Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus, and REV. According to the results, all IAVs showed a peak at the specific melting temperature (Tm; Fig. 1A), whereas other avian RNA viruses did not show this specific peak (Fig. 1B).

The sensitivity of SYBR-RT-qPCR was compared with that of TaqMan-RT-qPCR (Table 2). The detection limit of each RT-qPCR assay was defined as the highest dilution of RNA that provided a distinguishable amplification signal or melt curve over the negative control (Fig. 2). We measured the viral titers of PR8, mPR8-F-$C8_3A$, and mPR8-R-$A7_5G$-$C5_3$, and extracted RNA from each virus was diluted serially by 10-fold (10^{-1} to 10^{-10}). Each diluted RNA sample was examined by both SYBR-RT-qPCR and TaqMan-RT-qPCR. The detection limits of SYBR-RT-qPCR were 1.37, 1.69, and 7.89 EID_{50}, whereas those of TaqMan-RT-qPCR were 1.37, 1.69, and 78.9 for PR8, mPR8-F-$C8_3A$, and mPR8-R-$A7_5G$-$C5_3$, respectively (Table 2). The detection limits of PR8 and mPR8-F-$C8_3A$ were the same in both SYBR-RT-qPCR and TaqMan-RT-qPCR. The single mismatch in the eighth nucleotide from the 3' end of mPR8-F-$C8_3A$ was expected to reduce the efficiency of the amplification but did not affect the performance of PCR

significantly. However, the recombinant virus mPR8-R-$A7_5G$-$C5_3$ was detected more sensitively by SYBR-RT-qPCR than TaqMan-RT-qPCR. The recombinant virus mPR8-R-$A7_5G$-$C5_3$ has a mismatch in the fifth nucleotide from the 3' end which is quite closer to the 3' end as well as the mismatch in the seventh nucleotide from the 5' end which is insignificant.

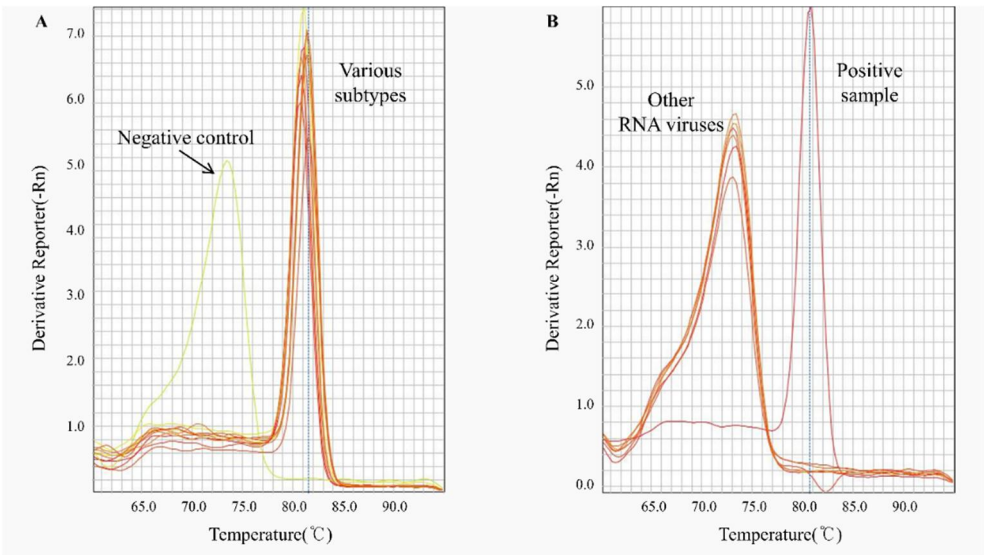

Fig. 4. Evaluation of sensitivity and specificity of the new generic primer set by SYBR-RT-qPCR

We detected various subtypes of influenza A viruses by SYBR-RT-qPCR for the evaluation of sensitivity (A) and examined various avian RNA viruses (a Newcastle disease virus vaccine strain, La Sota; field isolates of infectious bronchitis virus [SNU11045], infectious bursal disease virus [SNU16001], reticuloendotheliosis virus [SNU16008] for the evaluation of specificity (B). Negative control (DEPC-treated distilled deionized water).

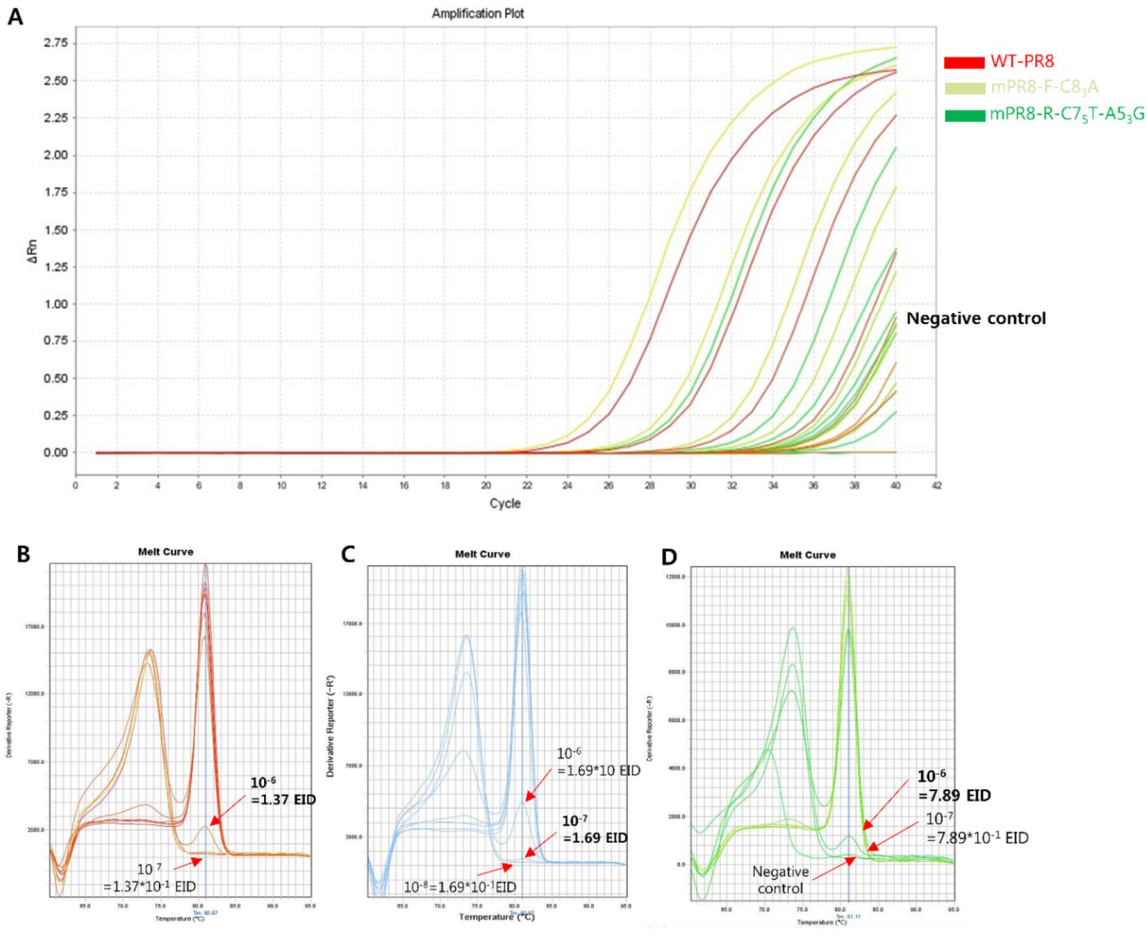

Fig. 5. Evaluation of the detection limit of Spackman's primer and new generic primer each for PR8, mPR8-F-C8$_3$A, mPR8-R-C7$_5$T-A5$_3$G

We evaluated the detection limit of commonly used Spackman's primer set (A) and the new generic primer set by SYBR-RT-qPCR for PR8 virus and recombinant PR8 viruses (Wild type-PR8(B), mPR8-F-C8$_3$A(C), mPR8-R-C7$_5$T-A5$_3$G(D)).

Table 2. Detection limit of two RT-qPCR assays

	Limit of detection[a] (EID_{50})[b]	
	SYBR-RT-qPCR	TaqMan-RT-qPCR
WT-PR8[c]	1.37	1.37
mPR8-F-$C8_3A$[d]	1.69	1.69
mPR8-R-$C7_5T$-$A5_3G$[e]	7.89	78.9

[a] Limit of detection was determined by the calculation of EID50 from the last dilution that was positive for the M gene.

[b] 50% of chicken embryo infective dose, EID_{50}/ml, geometric mean log_{10} titer

[c] Wild type PR8 (H1N1) virus

[d] Recombinant virus which has a mismatch in the eighth nucleotide from the 3' end

[e] Recombinant virus which has a mismatch in the fifth nucleotide from the 3' end as well as a mismatch in the seventh nucleotide from the 5' end

Optimization of the concentration of Triton X-100

The inactivation effect of Triton X-100 to influenza virus have already been reported, and we verified these findings and optimized the concentration of Triton X-100 by testing the infectivity of the 0028 strain in CEK cells after treatment with 0.3% and 0.5% Triton-X 100 (Table 3). The infectivity was evaluated by hemagglutination test. Intact 0028 strain retained infectivity until 10^{-5} dilution, whereas all Triton X-100-treated 0028 strains showed no infectivity. Therefore, 0.3% Triton X-100 was considered effective for inactivation of influenza virus. The biohazard of influenza virus contamination during viral RNP concentration experiment was prevented by using Triton X-100 with the concentration above 0.3%.

Table 3. Optimization of the concentration of Triton X-100 treatment

	Hemagglutination-positive wells[a]							
Dilution of virus[b]	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Virus only	4/4	4/4	4/4	4/4	4/4	1/4	0/4	0/4
0.3% Triton X-100	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
0.5% Triton X-100	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

The inactivation effect of Triton X-100 to influenza virus was evaluated by hemagglutination test using 1% chicken RBCs. Infectivity of the 0028 strain in CEK cells after treatment with 0.3% and 0.5% Triton-X 100 was tested. Treatment of Triton X-100 with concentration above 0.3% eliminated the infectivity of influenza virus.

[a] The supernatant medium of avian influenza virus-infected cells agglutinates chicken RBCs.

[b] 0028 strain (H9N2) was used as challenge virus.

Concentration of RNP in allantoic fluid and feces

Next, we compared the RNA amounts isolated from 150 μL of original allantoic fluid with purified RNPs from 10 mL original allantoic fluid containing different amounts of Triton X-100 (Table 4). We performed SYBR-RT-qPCR. The threshold cycle (C_T) of the original allantoic fluid was 24.95, whereas those of purified RNPs from 0.3, 0.5, 0.7, 1.0, and 1.5 mL Triton X-100-treated allantoic fluid were 21.93, 20.93, 19.95, 18.95, and 18.96, respectively. Thus, RNP purified by treatment with 1.0 or 1.5 mL Triton X-100 reduced the C_T by six cycles compared with 150 μL original allantoic fluid. These results indicated that the amount of purified RNA was 64-fold greater than that of the original unpurified sample. In addition, we compared the amount of RNA isolated from 150 μL of 10% fecal suspension with the amount of RNA isolated from purified RNP from 10 mL of a 5-mL Triton X-100-treated 10% fecal suspension. When using 5% Triton X-100, the difference in C_T value between the two samples was 4.17. Therefore, the concentrated sample contained 18-times more RNA than the unconcentrated sample.

Table 4. Comparison of viral RNA amounts before and after concentration of RNP by SYBR-RT-qPCR.

	Unconcentrated sample (C_T)[a]	Concentrated sample (C_T)					
Added Triton X-100 (10%)	0	0.3 mL	0.5 mL	0.7 mL	1.0 mL	1.5 mL	5 mL
Allantoic fluid (10 mL)	24.95	21.93	20.93	19.95	18.95	18.96	nt
10% suspension of feces (10 mL)	22.89	nt[b]	nt	nt	nt	nt	18.72

[a] C_T: Cycle threshold.

[b] nt: not tested.

Enhanced detection sensitivity by RNP concentration method

Furthermore, we verified the enhanced detection sensitivity of SYBR-RT-qPCR provided by concentration of viral RNP (Fig. 3). The detection abilities of SYBR-RT-qPCR combined with conventional RNA extraction method and SYBR-RT-qPCR combined with RNP concentration method were compared to confirm the practicality of RNP concentration method. From the fecal suspension sample containing 10^{-6} dilution of PR8 (H1N1) virus which is 1.33 x 10^3 EID_{50}/ml, it was diagnosed as positive by both of the RT-qPCR methods. However, the strength of the signal at the specific melting temperature was much higher in the concentrated sample than in the unconcentrated sample. In the case of the sample containing 10^{-7} dilution of PR8 virus which is 1.33 x 10^2 EID_{50}/ml, it was diagnosed as negative by the SYBR-RT-qPCR with conventional RNA extraction method, whereas it was diagnosed as positive by the SYBR-RT-qPCR with RNP concentration method. RNP concentration method reduced the detection limit more than 10-times which means smaller amount of viral particles can be detected using the same SYBR-RT-qPCR. In conclusion, our RNP concentration method could detect the virus below the detection limit of the conventional RNA extraction method.

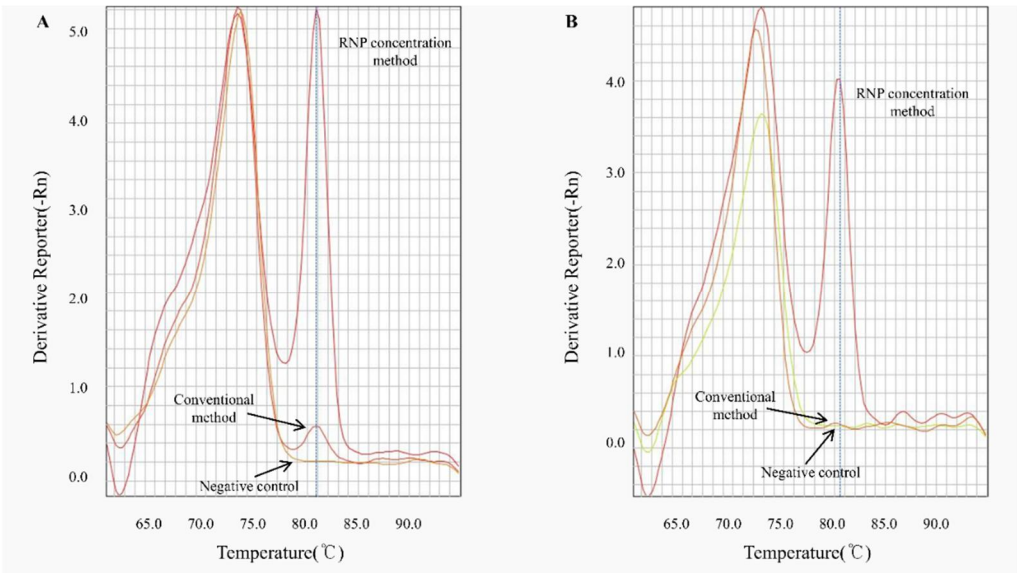

Fig. 6. Comparison of the detection limits of RNP concentration and conventional RNA extraction methods

PR8 virus was diluted by 10-fold, and RNA from 10^{-6} and 10^{-7} diluted samples was extracted directly or after RNP concentration. SYBR-RT-qPCR was performed, and melting curves were compared for the presence and signal intensity of the specific amplicon; 10^{-6} diluted sample (A) and 10^{-7} diluted sample (B), respectively.

Comparison of M gene-positive rate and diagnostic accuracy between SYBR-RT-qPCR and Taqman-RT-qPCR for detection of AIVs in fecal samples

To compare M gene-positive rate and diagnostic accuracy between SYBR-RT-qPCR and Taqman-RT-qPCR, the two RT-qPCR methods were performed on 120 fecal samples of migratory birds collected from the drainage basin of the the Geum-gang river, Chungnam, Korea (Table 5.). To describe the diagnostic characteristics of the two methods, virus isolation was used as a gold standard test. The sensitivity of both two RT-qPCR methods were evaluated to be 100% because all of the samples that were IAV-positive in the virus isolation method were diagnosed as positive by both methods and no false-negative sample was observed. The specificity was different between two methods showing 95.41% (270/283) in SYBR-RT-qPCR and 85.16% (241/283) in Taqman-RT-qPCR. More false-positive samples were observed in Taqman-RT-qPCR and therefore, the positive predictive value of SYBR-RT-qPCR was 43.48% (10/23), which is higher than 19.23% (10/52) of Taqman-RT-qPCR. The negative predictive values of both methods were same as 100%. In conclusion, the sensitivity was similar in both of the RT-qPCR methods, but the specificity and positive predictive value were significantly higher in SYBR-RT-qPCR.

Table 5. Comparison of M gene-positive rate and diagnostic accuracy between SYBR-RT-qPCR and Taqman-RT-qPCR for detection of AIVs in fecal samples

Sample label[a]	SYBR-RT-qPCR	Taqman-RT-qPCR	Virus isolation[b]
17-O_1-10	6/10 (60%)	9/10 (90%)	3/10 (30%)
17-N_1-80	1/80 (1.25%)	8/80 (10%)	1/80 (1.25%)
17-D_1-120	14/120 (11.67%)	32/120 (26.67%)	6/120 (5%)
18J_1-83	2/83 (2.41%)	3/83 (3.61%)	0/83 (0%)
Total	23/293 (7.85%)	52/293 (17.75%)	10/293 (3.41%)
Sensitivity	10/10 (100%)	10/10 (100%)	-
Specificity	270/283 (95.41%)	241/283 (85.16%)	-
Positive predictive value	10/23 (43.48%)	10/52 (19.23%)	-
Negative predictive value	270/270 (100%)	241/241 (100%)	-

Fecal samples were collected in the drainage basin of the the Geum-gang river, Chungnam, Korea. 5 fecal samples were pooled in a 15ml tube. A single tube of pooled samples was labeled as a single sample and subjected to a single reaction.

[a] Sample label: One single tube was labeled as a single sample according to the following rule; 'YY(year)-M(month)-No(number)'.

[b] Virus isolation: Virus isolation was used as a gold standard.

4. Discussion

The RNA-dependent RNA polymerases of IAVs have no proofreading function, and mutations occur randomly (Drake, 1993; Steinhauer et al., 1992). However, the mutation rates in the coding genes of eight segmented RNA genomes are different, and the internal genes are more conserved than HA and NA genes (Rambaut et al., 2008). Currently, M genes are frequent targets of molecular diagnosis due to the relatively high conservation of target regions among IAVs; however, the conserved region can change abruptly, causing false-negative results (Yang et al., 2014). The effects of mismatches between primers/probes and templates depend on the types and positions of mismatches. The mismatches between purines or pyrimidines and between incorrect pairs of purines and pyrimidines have different effects on the sensitivity of PCR-based diagnostics (Dieffenbach et al., 1993; Stadhouders et al., 2010; Ye et al., 2012). Furthermore, the effects of positions of mismatches in primers/probes, e.g., the 5′ end, middle region, and 3′-end, are also different. Although we checked the types and positions of primers, we only described the 100% identity rate of each primer for simplicity. Considering the comparable mutation rates of the M gene and the highly variable HA gene, the presence of highly conserved regions in the M gene is interesting (Rambaut et al., 2008). Thus, conserved regions with a

relatively high 100% identity rate, as shown in this study, may have less possibility of mutations than other regions.

Recent advancements have enabled specific amplicon to be detected by TaqMan probes, and small amplicons are preferable to reduce the nonspecific signal (Hulse-Post et al., 2005). Starick's primer set with a high 100% identity rate was expected to produce a relatively large amplicon (601 bp), and highly conserved sequences for TaqMan probes were absent between the forward and reverse primers. In Korea, Spackman's TaqMan-RT-qPCR has been used for molecular diagnosis of AIVs, but the relatively low 100% identity rates of both primers were unexpected. In addition, the target region of the TaqMan probe should be conserved for sensitive and specific fluorescence emission, although mutations are inevitably encountered (Yang et al., 2014). Therefore, we designed improved forward and reverse degenerate primers for SYBR-RT-qPCR that did not require additional conserved regions for probes.

The similar sensitivities of SYBR-RT-qPCR and TaqMan-RT-qPCR for PR8 and mPR8-F-C8$_3$A may reflect the lack of effects of primer mismatches at this position. However, in comparison with SYBR-RT-qPCR, two-nucleotide mismatches in the reverse primer clearly reduced the sensitivity of TaqMan-RT-qPCR. Considering the single nucleotide mismatch of Spackman's reverse and forward and reverse primers to corresponding regions of PR8 and mPR8-F-C8$_3$A, respectively, such

mismatches may not affect the sensitivity of TaqMan-RT-qPCR. However, the different sensitivity may be attributed to the two-nucleotide mismatches between Spackman's reverse primer and the corresponding region of mPR8-R-$A7_5G$-$C5_3$. The frequency of wild IAVs with the same mismatches in our database was 9.3% (320/3,441), and the sensitivity of TaqMan-RT-qPCR may decrease in specimens from randomly collected pooled samples. IAVs often exhibit changes in genome sequences; therefore, regular primer and probe quality assessments by comparing complementarity with newly added sequence data should be conducted.

Although TaqMan-RT-qPCR is highly sensitive and specific, we encountered an unexpected problem. The fluorescence signals were occasionally amplified even in negative controls and in some samples containing high concentrations of non-IAV-related RNA. Even though the signals increased during late rounds of the thermocycles, misleading false-positive results were observed (data not shown). To understand the false-positive results, we visualized the amplicons using 2% agarose gel electrophoresis; however, the size of the amplicon generated by TaqMan-RT-qPCR was only 101 bp, and specific and nonspecific amplicons were difficult to distinguish. However, our SYBR-RT-qPCR did not cause any false-positive results, and the specific amplicon was easily distinguished from nonspecific primer dimers based on analysis of the melting temperature (data not shown). Nonspecific amplicons with different melting

temperatures showed smaller sizes on electrophoresed agarose gels. Furthermore, SYBR-RT-qPCR did not require a probe, for which mismatch may decrease the sensitivity of diagnosis. Thus, our SYBR-RT-qPCR was expected to be useful for the molecular detection of IAVs with regard to sensitivity and specificity.

To confirm the improved sensitivity and specificity of our new SYBR-RT-qPCR method, we performed our new SYBR-RT-qPCR and Taqman-RT-qPCR on the 293 fecal samples collected from the drainage basin of the the Geum-gang river (Table 5.). The sensitivity and negative predictive value were same as they were 100% in both of the RT-qPCR methods. However, the specificity and positive predictive value of our SYBR-RT-qPCR were 95.41% and 43.48%, respectively, which were significantly higher than those of Taqman-RT-qPCR, 85.16% and 19.23%, respectively. The differences between the two RT-qPCR methods were mainly because of the false positive results. The fecal samples that was positive in Taqman-RT-qPCR and negative in virus isolation were probably including some copies of M gene but none of live AIVs. In the aspect of virus isolation, Taqman-RT-qPCR method diagnosed more negative samples as positive and it lowered the efficiency of the subsequent processes. Therefore, our new primer set with SYBR-RT-qPCR method with great specificity can increase the efficiency of the entire process for the surveillance of avian influenza.

AIVs are shed in saliva, nasal secretions, and feces of infected birds. Among these samples, feces are easily collected. Wild birds and domestic ducks infected by highly pathogenic AIVs and chickens infected by weakly pathogenic AIVs may release virus particles into feces without any clinical manifestations (Hulse-Post et al., 2005; Webster et al., 1978). During the early and convalescent period of infections, the titers of shed virions are relatively low, and pooling of samples to reduce testing numbers may dilute virions more (Spackman et al., 2013). Several methods to concentrate virus particles have been reported; however, previous studies have only captured and separated viral particles. Dhumpa *et al*. successfully applied anti-NP antibody-bound MBs to fecal virus purification and performed direct RT-qPCR without RNA purification (Dhumpa et al., 2011). They did not use Triton X-100 for envelope solubilization to expose RNPs. They found similar sensitivities to conventional RT-qPCR employing RNA extraction procedures. According to our results, our RNA concentration method combined with RNA extraction was more sensitive than conventional RT-qPCR. Therefore, both optimized Triton X-100 treatment and RNA purification steps may yield different results. Triton X-100 treatment may also improve biosecurity during fecal RNP extraction, and simple RNA extraction methods requiring only a few minutes have recently been developed (Colavita et al., 2017; Lina et al., 2000; Stead et al., 2012).

Thus, in this study we improved the generic primer set and provided

an improved protocol to concentrate and purify RNPs from specimens for more sensitive IAV detection. Because certain mismatches in primers and probes can affect the sensitivity of PCR-based molecular diagnosis, regular primer quality assessment and sequence tuning should not be ignored.

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국 문 초 록

개선된 제네릭 프라이머와 새로운 바이러스 RNP 복합체 농축을 통한 A형 인플루엔자 바이러스 분자적 진단 기법 확립

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수의학과 수의병인생물학 및 예방수의학 전공

검체 내 표적 유전자의 복제 수 뿐만 아니라 프라이머와 주형 사이의 불일치의 비율과 위치 또한 PCR 기반의 진단법들의 민감도에 영향을 줄 수 있다. 오늘날, A형 인플루엔자 바이러스를 검출하기 위한 다양한 제네릭 프라이머 세트들이 보고되어 있다. 그러나, 아직까지 그들의 불일치 비율은 포괄적으로 평가된 적이 없다. 이번 연구에서 우리는 3, 441개의 matrix 유전자의 coding

region과 기보고된 프라이머 세트들 사이의 염기 서열 동일성을 평가하였다. 결과에 따르면, 비교 대상이 되었던 프라이머들 중에 100% 일치도를 보이는 것은 하나도 존재하지 않았다. 따라서, 우리는 비교 대상이 되는 전체 유전자들 중 98% 이상의 유전자들과 염기서열이 100% 일치하는 새로운 degenerate 프라이머 세트를 제작하였고, SYBR 방식의 실시간 중합효소연쇄반응을 사용했을 때 프라이머의 증폭 효율을 TaqMan probe 방식의 실시간 중합효소연쇄반응과 비교하였다. 그리고 새 프라이머를 사용한 SYBR 방식과 기존의 Taqman 방식 각각의 진단 한계를 A형 인플루엔자 바이러스의 RNA를 10진 희석한 일련의 시료에서 평가하였다. 실험 결과에 따르면, 야생형의 A형 인플루엔자 PR8 바이러스에 대해서는 두 RT-qPCR 방법이 1.37 EID_{50}의 동일한 진단 한계를 나타냈으나, 3’ 의 5번째 염기에서 불일치를 갖도록 제작된 재조합 PR8 바이러스에 대해서는 새로운 프라이머를 사용한 SYBR 방식이 7.89 EID_{50}의 진단한계를 나타내어 78.9 EID_{50}의 진단한계를 나타낸 Taqman 방식에 비해 10배 향상된 분석적 민감도를 보이는 것을 확인하였다. 추가적으로, 새로운 프라이머를 사용한 SYBR 방식과 기존의 Taqman 방식의 진단 민감도, 진단 특이도, 양성 예측도를, 금강 유역에서 채취한 293개의 철새 분변 시료에서 비교 평가하였다. 두 방식의 진단 민감도는 100%로 동일하였으나, SYBR 방식의 진단 특이도와 양성예측도가 각각 95.41%와 43.48%로 Taqman 방식의 85.16%와 19.23%보다 우수한 것을 확인하였다. 나아가, 요막강액과 분변 희석액에서 Triton X-100,

anti-NP 마우스 단클론항체, anti-Mouse Ig 항체가 결합된 자성 구를 이용한 바이러스의 RNA-핵단백질 복합체 농축 방법을 통하여 SYBR 기반의 실시간 중합효소연쇄반응의 민감도를 성공적으로 향상시켰다. 농축 방법을 이용하여 viral RNP를 농축정제했을 때와 기존의 RNA 추출 방법으로 viral RNA를 추출하였을 때, 각각 용출된 RNA 샘플 내의 viral RNA 농도를 비교한 결과, 요막강액에서 64배, 분변 희석액에서 18배의 농축 효율을 확인하였다. 추가적으로, 기존의 RNA 추출 방법의 진단 한계 이하의 농도에서도 RNP 농축 방법을 이용할 시 바이러스 검출이 가능한 것을 확인하였다. 따라서, 개선된 제네릭 프라이머 세트와 RNP 농축 방법의 적용은 A형 인플루엔자 바이러스의 민감한 진단에 유용할 것으로 기대된다.

주요어: A형 인플루엔자 바이러스, matrix 유전자, 제네릭 프라이머, 실시간중합효소연쇄반응, 바이러스 RNP 복합체 농축
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